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Shigella sonnei vaccine candidates WRSS2 and WRSS3 are as immunogenic as WRSS1, a clinically tested vaccine candidate, in a primate model of infection

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ABSTRACT

Shigella causes diarrhea and dysentery through contaminated food and water. *Shigella sonnei* live vaccine candidates WRSS2 and WRSS3 are attenuated principally by the loss of VirG(lcsA) that prevents bacterial spread within the colonic epithelium. In this respect they are similar to the clinically tested vaccine candidate WRSS1. However, WRSS2 and WRSS3 are further attenuated by loss of *senA*, *senB* and WRSS3 also lacks *msbB2*. As previously shown in cell culture assays and in small animal models, these additional gene deletions reduced the levels of enterotoxicity and endotoxicity of WRSS2 and WRSS3, potentially making them safer than WRSS1. However the behavior of these second-generation VirG(lcsA)-based vaccine candidates in eliciting an immune response in a gastrointestinal model of infection has not been evaluated. In this study, WRSS2 and WRSS3 were nasogastrically administered to rhesus monkeys that were evaluated for colonization, as well as for systemic and mucosal immune responses. Both vaccine candidates were safe in rhesus monkeys and behaved comparably to WRSS1 in bacterial excretion rates that demonstrated robust intestinal colonization. Furthermore, humoral and mucosal immune responses elicited against bacterial antigens appeared similar in all categories across all three strains indicating that the additional gene deletions did not compromise the immunogenicity of these vaccine candidates. Based on data from previous clinical trials with WRSS1, it is likely that, WRSS2 and WRSS3 will not only be safer in human volunteers but will generate comparable levels of systemic and mucosal immune responses that were achieved with WRSS1.

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1. Introduction

Shigellosis is a disease characterized by an acute inflammatory colitis elicited by bacterial invasion of the intestinal epithelium [1]. The etiological agent is *Shigella*, a gram negative bacteria consisting of 4 serogroups, *Shigella dysenteriae*, *Shigella flexneri*, *Shigella boydii* and *Shigella sonnei*, that is also referred to as *Shigella* group A, B, C and D strains, respectively [2]. The bacteria target mainly the distal colon, and the infection results in diarrhea, fever, dysentery and considerable gastrointestinal and constitutional symptoms. The low infective dose of 10–100 bacteria, and high transmissibility makes control of *Shigella* infections very challenging, particularly in less developed regions of the world. Epidemiological data, as well as limited studies in animals, have indicated that a vaccine against *S. flexneri* 2a, 3 and *S. sonnei* would protect against 80% of shigellosis seen worldwide [3,4]. *S. sonnei* predominates in the United States and in other developed countries while *S. flexneri*

2a and 3, are among the most common serotypes circulating in the developing world. Infection with a particular serotype provides homologous but not heterologous protection, affirming the important role played by the bacterial lipopolysaccharide (LPS) in providing a target for serotype-specific protection. In addition, several conserved protein antigens encoded on the large virulence plasmid, such as the Ipa antigens (IpaA, B, C and D) are also being targeted for an optimal response [5–9].

Experiments in rabbit ileal loops and in rhesus monkeys have shown that shigellae are initially taken up by antigen sampling microfold (M) cells that are located in the follicle-associated regions of the epithelium [10–12]. Transcytosis of the bacteria from the M cells into the underlying follicle-associated immune effector cells facilitates subsequent basolateral invasion of adjacent enterocytes by a mechanism of induced phagocytosis mediated by the Ipa proteins [13]. After escaping from the endocytic vacuole, the bacteria multiply within the cytosol of enterocytes and recruit host cell actin to form a cytoskeleton-based motor that results in bacterial spread from one cell to another [14–16]. During this movement, the bacteria impinges on the inner face of the cytoplasmic membrane resulting in rigid protrusions that require the participation of cad-

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herins and other tight junction proteins [17]. Subsequent lysis of the internalized protrusions by the protein IcsB releases shigellae into the cytoplasm of contiguous epithelial cells [18,19].

Bacterial movement within the cell and from cell to cell requires the functioning of a critical bacterial surface protein, VirG(or IcsA), which assembles host cell actin filaments at the distal pole of the bacterium formed during septation [15,20–22]. Loss of VirG(IcsA) eliminates actin recruitment and significantly attenuates bacterial virulence as assayed in cell cultures and in animal models [11,23–29]. Several vaccine candidates of different serotypes lacking VirG(IcsA), such as SC602, WRSS1 and WRSD1, have been successfully tested in human volunteers and proven to be safe at low doses and immunogenic [30–34]. In the case of the *S. flexneri* 2a vaccine candidate SC602, vaccinated volunteers subsequently challenged with a virulent strain were protected against severe disease, providing proof of concept for this strategy of attenuation [30]. Similar strong immune responses were also seen during Phase 1 trials with *S. sonnei* vaccine candidate WRSS1, that were predictive of a protective response [31,33].

Approximately 15–20% of the volunteers who received 10^4 CFU of SC602, WRSS1 and *S. dysenteriae* WRSD1, showed some reactogenic symptoms such as mild and transient diarrhea and fever that increased at higher doses. New second-generation vaccine candidates, such as *S. sonnei* strains WRSS2 and WRSS3, have been developed to minimize reactogenicity and provide a wider window of safety [35]. These candidates not only lack VirG(IcsA) but also have deletions in known enterotoxin genes (*senA*), putative homologs of known enterotoxin genes (*senB*) and in some cases lipid A acyl transferase gene *msbB2*, whose loss is intended to reduce fever by reducing LPS endotoxicity [35]. Although cell culture assays and small animal models have demonstrated that these new candidates have reduced reactogenicity, one concern that remained was whether these newer, more attenuated derivatives will colonize the gastrointestinal tract (GIT) to the same extent as did WRSS1. A relevant model to test vaccine colonization is the non-human primate model, since rhesus monkeys are the only other animals that succumb to natural infections with *Shigella* and get symptoms of shigellosis similar to humans [6,36–40].

WRSS2 and WRSS3 were administered to small groups of rhesus monkeys and evaluated for safety and colonization [29]. Blood and fecal samples were collected for immunogenicity assays including both systemic and mucosal responses measured against LPS and Invaplex, a complex of LPS and Ipa proteins [9,41]. The results demonstrate that in spite of increased attenuation, both vaccine candidates were able to elicit an immune response that was similar to that obtained with WRSS1. Based on these data, WRSS2 and WRSS3 are expected to cause fewer reactogenic symptoms in human volunteers, yet maintain the robust immunogenicity profile associated with WRSS1.

2. Materials and methods

2.1. Animals and bacterial strains

All animals were housed in individual cages in the WRAIR monkey colony. They were adult male, Indian strain rhesus macaques (*Macaca mulatta*). In this study, 3 groups of 5 animals each were randomly assigned and administered either WRSS1, WRSS2 or WRSS3 (Fig. 1) [29]. These studies with *Shigella* were carried out under a WRAIR IACUC-approved protocol.

2.2. Oral vaccination procedures in rhesus monkeys and safety evaluation

Sedated monkeys were inoculated nasogastrically with 4×10^{10} CFU of freshly harvested WRSS2 and WRSS3 preceded by 20 ml

of sodium bicarbonate solution to neutralize gastric acidity. The method of vaccine preparation, administration and safety evaluation of vaccinated monkeys has been previously described [29].

2.3. Blood sample collection

Blood samples were collected before immunization (day –4) and on days 4, 7, 9, 14, 28 and analyzed for antigen-specific antibody secreting cells (ASCs). Serum antibody titers were determined for samples collected on day –4, 14 and 28.

2.4. Serology

Serum antibody endpoint titers were determined by ELISA as previously described [42]. In brief, Immunolon 1B 96-well microtiter plates were coated overnight at 4 °C with 100 µl of *S. sonnei* LPS, IpaB, IpaC, or *S. sonnei* Invaplex 50 diluted to 10, 1, 2, or 0.5 µg/ml in carbonate coating buffer (pH 9.8). Plates were blocked for 30 min with 2% casein in Tris–saline buffer. Plasma samples collected on day –4, 0, 14, and 28 were diluted in 2% casein and titrated across the plate in duplicate using 2-fold serial dilutions and incubated for 2 h [42]. The plates were washed 4 times with PBS containing 0.05% Tween 20 (PBS-T) and incubated for 1 h with goat-anti-human IgG-AP or goat-anti-human IgA-AP. Bound antigen-specific antibody was detected by incubating the plates for 30 min with phosphatase substrate. The optical density at 405 nm was measured with a plate reader [42]. The endpoint titer was defined as the reciprocal of the last dilution of sample that produced an OD value of 0.2. A responder was defined as having a ≥ 4 -fold increase in titer over baseline.

2.5. Antibody-secreting cell (ASC) assay

Peripheral blood mononuclear cells were isolated by Ficoll density gradient and cryopreserved in DMSO freezing medium as described previously [43]. *S. sonnei* LPS and Invaplex-50 antigen-specific IgA and IgG-secreting cells in circulation by ELISPOT using goat anti-human antibodies as previously described [46]. Individual animal data are presented as maximum number of ASC/ 10^6 PMBC. Responders were defined as ≥ 5 antigen-specific ASCs/ 10^6 PMBCs.

2.6. Rectal lavage sample collection and analysis

A sterile, disposable, plastic 8 in. long infant feeding tube was inserted approximately 4–5 cm into the rectum of the monkeys. The rectum was flushed three times with 6 ml of sterile PBS using a 6 ml syringe attached to the sterile infant feeding tube. The rectal lavage sample in PBS was aspirated back into the 6 ml syringe. These lavage samples were centrifuged at 4 °C for 20 min at $1500 \times g$, and the supernatant was filtered through 0.2 µm centrifuge filter tubes by centrifugation at 4 °C for 30 min at $1500 \times g$. Protease inhibitor AEBSF was added. The samples were aliquoted and stored at –80 °C until use. Aliquots of the lavage samples were used in an ELISA assay against *S. sonnei* LPS and Invaplex.

2.7. Fecal IgA

Total and antigen-specific fecal IgA were determined by ELISA using human-specific reagents as described [43–45]. The antigen-specific endpoint titers were determined, and the final titer for each animal was adjusted to 10 µg/ml of total IgA. Animals showing < 2 µg/ml of total IgA were not included for antigen-specific evaluations. The data for individual animals are presented as maximum fold increase over the base line. A responder was defined as having a ≥ 4 -fold increase in titer over baseline.

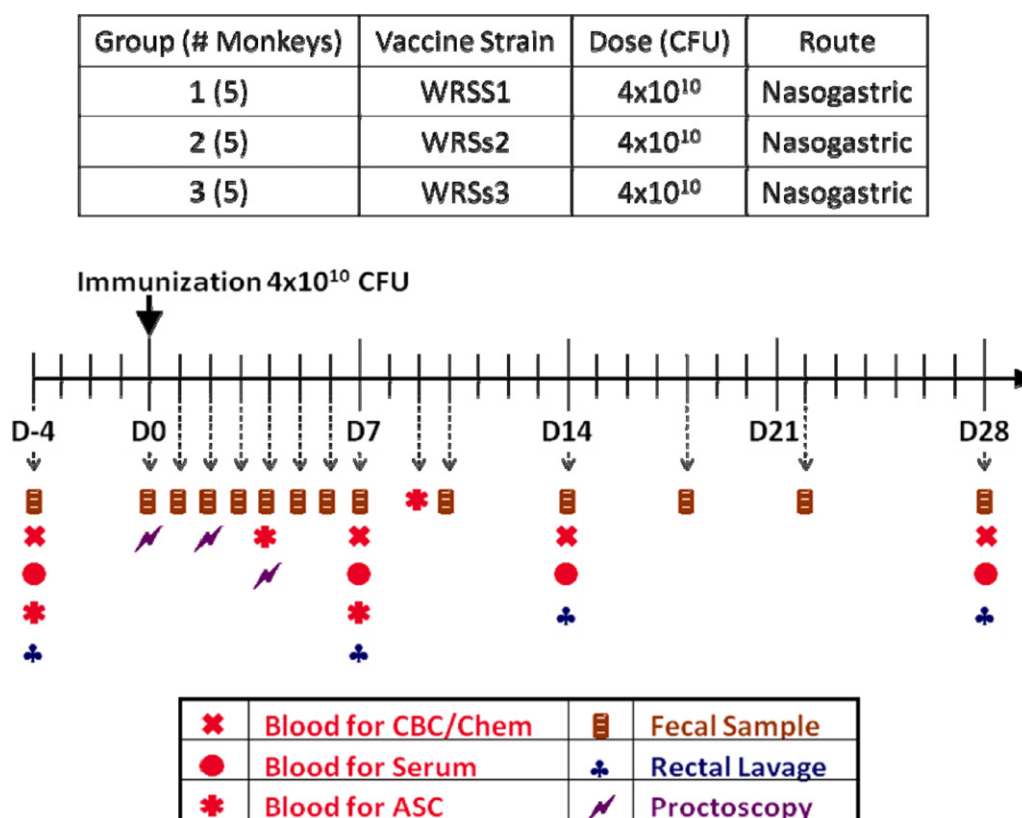


Fig. 1. Rhesus monkey study outline for evaluating safety and immunogenicity of WRSS1, WRSs2 and WRSs3. Fifteen adult male rhesus macaques, ranging in age from 8 to 21 years, obtained from the WRAIR animal colony were randomized to receive one of three different *S. sonnei* vaccine candidates as shown in the panel. The lower panel shows schedules of procedures and various samples taken for evaluation of excretion and immune responses. Animals were immunized with the vaccine candidates at D0 with freshly harvested cultures after ingestion of 20 ml of bicarbonate to neutralize gastric acidity as described in Section 2 [29]. These studies were done under an IACUC-approved monkey protocol.

2.8. Statistical analysis

Statistical analysis for serum antibody responses used Prism 4 for Macintosh (Graphpad Software, Inc). Log-transformed endpoint titers were analyzed using a two-way analysis of variance with a Bonferroni post-test.

3. Results

3.1. Clinical symptoms and stool cultures of rhesus monkeys administered WRSS1, WRSs2 and WRSs3

WRSs2, WRSs3, and WRSS1, were nasogastrically administered to groups of 5 rhesus monkeys after sedation. Except for occasional soft stool, no signs of diarrhea or dysentery were observed in any group [29]. Hematologic responses have been previously described and endoscopic exams of the rectum and distal colon did not reveal evidence of any vaccine-induced lesions [29]. All three vaccine candidates colonized the GI tract (presumptive evidence of colonization) and were excreted to similar extents, with culture positive stools in all but one monkey (in the WRSs2 group) seen for 2 days [29]. Thereafter, the duration of vaccine shedding in all 3 groups was gradually reduced although some animals showed culture positive stools till day 7. PCR with *ipaH*-specific primers was carried out on DNA extracted from stools on days 3, 5, 7, 10, in particular, to determine whether a second method of identification could be extended to culture negative samples. However, a consistent pattern of PCR-positive amplification, even with ribosomal primers, was not observed with every template indicating that

the reaction was being inhibited. However, in all 3 groups, some of the culture negative samples did indicate a correct sized PCR amplified band, in four cases, up to day 10 (Table 1). Although most monkeys shed the vaccine strains, a direct correlation between culture positive or PCR positive stools and the magnitude of a particular immune response could not be determined in this study.

3.2. Serum antibody responses after vaccine administration

Serum IgG and IgA endpoint titers specific for *S. sonnei* LPS, *S. sonnei* Invaplex 50, IpaB, or IpaC, were determined by ELISA. Robust levels of *S. sonnei* Invaplex 50 and IpaB-specific serum IgG were induced after immunization with WRSS1, WRSs2 or WRSs3. Greater than 80% of the animals in each group seroconverted by day 14 and maintained the same level on day 28 (Fig. 2 and Table 1). No significant differences were detected between animals receiving WRSS1 and WRSs3. Levels of Invaplex-specific IgG and IpaB serum IgA were significantly higher in animals immunized with WRSs2 as compared to WRSs3 ($p < 0.05$) based on endpoint titer analysis on both day 14 and 28 (Fig. 2). Also, the IpaB and IpaC-specific serum responses were significantly higher in animals receiving WRSs2 as compared to WRSS1 (Table 1 and Fig. 2). However, the peak fold titers to the different antigens were similar in the three groups (Table 1). Interestingly, few animals in any of the three vaccine groups seroconverted to *S. sonnei* LPS, with conversion rates of $\leq 40\%$ (Table 1). For most antigens, peak serum IgG and IgA titers were detected in samples collected on day 14 (Fig. 2).

Table 1
Shigella antigen-specific immune responses and colonization in rhesus monkeys after nasogastric administration of WRSS1, WRSs2 and WRSs3.

Vaccine	ID	Peak antigen-specific ASC/10 ⁶ PBMC				Fecal antibody (peak fold-rise)		Serum antibody (peak fold-rise)								Excretion/colonization (days after infection)	
		Anti-LPS		Anti-Invaplex		Anti-LPS	Anti-Invaplex	Anti-LPS		Anti-Invaplex		Anti-IpaB		Anti-IpaC		Culture	PCR ^b
		IgA	IgG	IgA	IgG			IgA	IgG	IgA	IgG	IgA	IgG	IgA	IgG		
WRSS1	B34Z	0	1	3	7	1	84	1	2	8	32	2	8	4	2	2	3
	F942	29	11	0	16	9	20	1	1	4	32	1	16	4	4	4	ND
	JKA	1	0	5	4	1	13	1	1	8	8	2	32	1	2	6	7
	JPT	0	0	11	0	1	2	1	1	4	8	1	8	2	2	6	5
	TAB	9	0	25	6	2	74	1	2	4	8	2	32	1	2	7	10
Responder rate ^a		40%	20%	60%	60%	20%	80%	0%	0%	100%	100%	0%	100%	40%	20%		
WRSs2	20H	101	6	113	8	66	17	4	8	4	16	2	32	4	8	2	10
	694Z	0	0	1	9	1	174	1	1	4	16	4	4	4	16	7	7
	89–129	0	0	0	2	1	5	1	1	1	4	1	4	2	4	3	10
	C42Z	2	3	1	12	15	6	1	4	16	4	2	164	2	16	2	7
	CJ2W	0	7	0	3	1	46	1	1	32	32	16	2	32	64	0	ND
Responder rate		20%	40%	20%	60%	40%	100%	20%	40%	80%	100%	40%	80%	60%	100%		
WRSs3	89–155	0	1	1	3	1	37	1	1	4	32	4	128	16	4	4	7
	A14Z	0	0	2	0	1	3	1	1	1	2	1	4	1	4	2	7
	BVF	2	1	5	19	8	10	1	1	2	16	2	64	4	2	5	7
	J778	35	5	35	16	2	22	4	2	4	32	1	32	8	32	5	7
	TAJ	0	9	2	5	1	2	1	1	1	8	1	32	2	2	2	10
Responder rate		20%	40%	20%	60%	20%	60%	20%	0%	40%	80%	20%	100%	60%	60%		

^a Responder definitions: antigen-specific ASC: ≥ 5 ASCs/10⁶ PBMCs; fecal and serum antibody: \geq four-fold increase over baseline.

^b PCR done on DNA extracted from stools on days 3, 5, 7, 10 [29]. The numbers indicate PCR positive stool days.

ND, not detected.

Numbers in bold indicate responders.

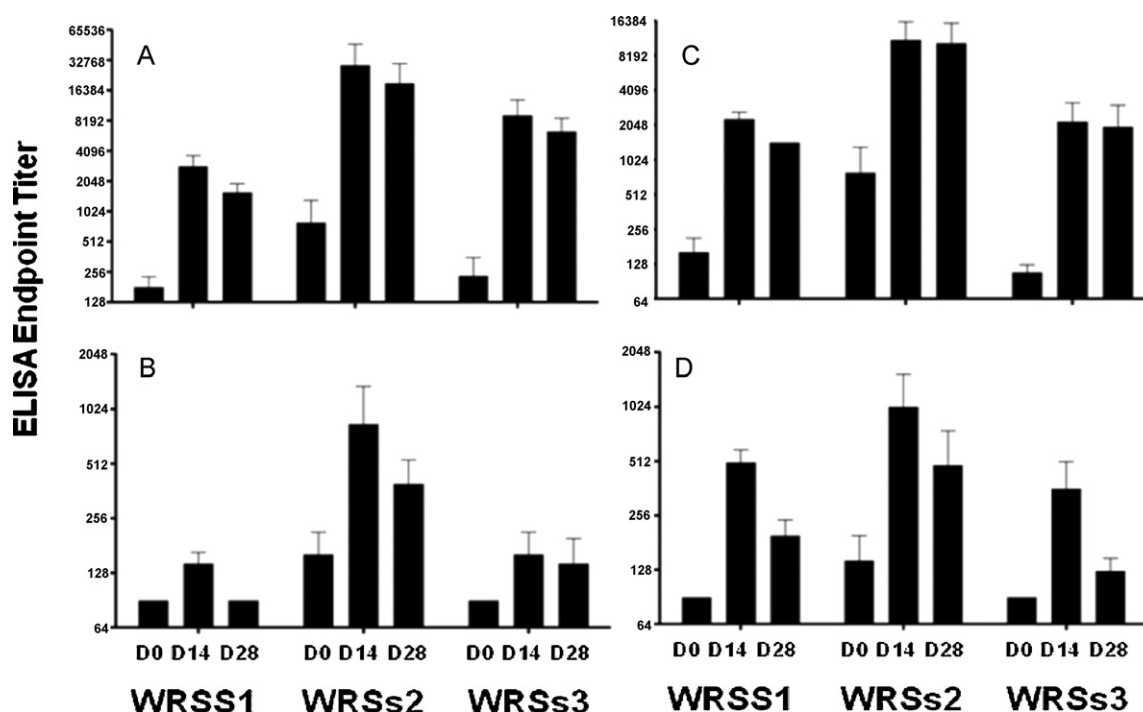


Fig. 2. Serum antibody responses to IpaB (Panels A and B) and to Invaplex (Panels C and D) in rhesus monkeys after oral administration of *S. sonnei* vaccine candidates WRSS1, WRSS2 and WRSS3. Panels A and C reflect IgG values and panels B and D indicate IgA to the respective antigens.

3.3. Mucosal responses in rhesus monkeys

The mucosal immune response to *Shigella* LPS, and Invaplex-50 antigens are summarized in Table 1. Both ASC and fecal IgA responses to Invaplex were higher than to LPS. At least 2 of 5 animals in each vaccine group had IgA- or IgG-ASC specific to LPS and at least 3 of 5 animals had IgA- or IgG-ASC specific to Invaplex in circulation. The magnitude of the ASC responses was variable with the highest values seen for IgA responses. Generally, only one animal in each group responded with both an IgA and an IgG ASC response to LPS and to Invaplex. In the WRSS3 group two animals responded with both an IgA and an IgG ASC response to Invaplex.

Fewer animals developed LPS-specific fecal IgA responses and the fold increase over the base line among the responders was variable (range 8.2–66.0). Irrespective of the vaccine strain used for immunization, similar levels of total fecal IgA in stool were detected in all animals prior to vaccination or any time after vaccination. Compared to LPS, fecal IgA response rates to Invaplex antigens were higher with 60–100% of the animals indicating a positive response (Table 1). The peak fecal IgA responses were detected on study days 7–9 for most of the animals (except one animal in WRSS1 group whose fecal IgA response peaked at day 14). Among the responders, the mean fold rise in fecal IgA to Invaplex was 48, 50, respectively (Table 1).

Rectal lavage samples were analyzed for IgA and IgG to *S. sonnei* Invaplex and LPS in an ELISA assay as described for serum samples. In general, a detectable response was seen only in a few animals. A 4-fold rise in IgA titer to Invaplex was detected in only one animal (B34Z) in the WRSS1-immunized group and two animals (694Z and C42Z) in the WRSS2 group at day 7. A 2-fold rise in IgA titer to LPS was seen in one animal in the WRSS1 immunized group (F94Z) and two animals in the WRSS2 group (694Z and C42Z) on days 7 and 14.

4. Discussion

Previous studies with rhesus monkeys have shown that the infective dose for *Shigella* is in the range of 10^{10} virulent organisms. Once infected with a wild-type strain, monkeys can experience diarrhea alone, diarrhea and dysentery or only dysentery within 24 h after challenge [47]. Monkeys with dysentery alone have net colonic secretion and in the most severe cases have extensive colitis accompanied by a large number of bacteria on the surface of the colonic epithelium and the more luminal crypt cells [48]. In these examples, the surface epithelium showed evidence of abnormal extrusion and shedding of cells, and as the defect widened, it was covered with a fibrinous exudates or by leucocytes [48,49]. The inflammatory response diminishes from the luminal surface to the submucosa. The intensity of the inflammatory response corresponded to the degree and depth of bacterial penetration [48,50]. If left untreated, monkeys with dysentery can sometimes die of the disease. Thus, rhesus monkeys have provided a unique and physiologically relevant animal model system to test the acute phase of bacillary dysentery and to evaluate safety and immunogenicity of live *Shigella* vaccines [6,42,47,51].

In previously described studies, the *S. sonnei* virulent strain 53G, given at a dose of 2×10^{10} CFU in 20 ml of brain–heart infusion media, induced diarrheal illness in 8 of 12 rhesus monkeys [6]. WRSS1, WRSS2 and WRSS3, given at the same dose, induce minimal overall clinical abnormalities [29]. Infrequent soft stools were the most common finding in all groups but no diarrheal symptoms were noted. Since a control group was not included in this study, the presence of soft stools cannot be directly attributed to vaccine administration. No animals exhibited fever. Like WRSS1, the two new candidates, WRSS2 and WRSS3 colonized the GIT successfully and to the same extent, with minor clinical side effects [29].

The immune response generated in rhesus monkeys after a single dose of WRSS2 or WRSS3 was comparable to WRSS1 with more

animals in each group responding to Invaplex than to LPS. This is a promising observation since WRSS1 was also very immunogenic in human volunteers. 80–100% of the monkeys in all three groups had ≥ 4 -fold IgG serum antibody titers to *S. sonnei* Invaplex as well as to the IpaB protein and the peak-fold titers in all 3 groups were of comparable magnitude. The same was generally true of the fecal IgA response rates to Invaplex (60–100%) and the IgG ASC response rates to Invaplex (60%) in the 3 groups. The higher rate of responses to Invaplex, as compared to LPS alone, in all categories of immune responses measured is probably because the Invaplex is a combination of LPS and protein antigens (mostly IpaB and IpaC). Additional doses of the vaccines would likely improve the response rates to LPS. Indeed, three vaccinations with SC602 in rhesus monkeys elicited significant IgG and IgA responses against the homologous LPS and against a water extract of *Shigella* bearing the Ipa proteins (unpublished data). A follow-up study in rhesus monkeys with multiple doses of the *S. sonnei* candidates would be required to validate this observation.

It is interesting to note that the serum IgG antibody response to the two Ipa proteins, particularly to IpaB, were quite significant (80–100% for IpaB in all 3 groups). The IpaB and IpaC proteins (as well as IpaD, IpaA and VirG(IcsA), are surface localized highly conserved antigens present in all virulent serotypes of *Shigella* [7,42]. Both IpaB and IpaC are critical for the invasive phenotype of the bacteria and serum and mucosal immune responses to both proteins can be detected after infections in monkeys and in humans [30,42,44,52–54]. Serum antibodies to Ipa proteins, as well as LPS, have been shown to inhibit invasion of *Shigella* in cultured cells [55,56]. An artificial Invaplex, which contains purified LPS mixed with purified IpaB and IpaC is highly protective in guinea pigs. Although the specific immune mechanisms that confer protection against disease is still unclear, natural infections as well as vaccination/challenge studies suggests that, in addition to LPS, a strong response to the Ipa proteins, may be requisite for long-term protection against shigellosis [9,57]. In fact, in a Swedish cohort of volunteers, convalescent sera with antibodies to LPS and the Ipa proteins were seen six months after infection [58].

The only VirG(IcsA)-based vaccine that has undergone a Phase 2 challenge study is the *S. flexneri* 2a vaccine strain SC602 [30]. Certain parallels can be inferred from the behavior of SC602 in rhesus monkeys and in humans. Previous vaccination studies with SC602 in rhesus monkeys (1×10^{11} CFU dose per monkey, 3 doses given on days 0, 10 and 20) indicated that, all monkeys shed the vaccine strain during the first 24 h after each inoculation with significantly reduced shedding during later exposures (unpublished data). When immunized and control monkeys were challenged with a virulent strain 2457T, a small proportion (12.5%) of vaccinated as well as control animals excreted dysenteric stools within 24 h. Subsequently, the cumulative proportion of control animals excreting dysenteric stools increased significantly (>80% by day 6), and one animal died on day 4. However, the proportion of vaccinated animals experiencing disease was substantially unchanged after the first day of observation. Overall, the data indicated that SC602 in rhesus monkeys was associated with 75% protection against overt dysentery (unpublished data). Significant serum antibody responses to LPS and the Ipa proteins (in water extract) were seen after three inoculations although other parameters of immunogenicity were not measured in this study (unpublished data). When SC602 was administered to human volunteers at a single dose of 10^4 CFU, 92% of the volunteers shed the vaccine for several days but at higher doses the vaccine candidate was more reactogenic [30]. The excretion rates were indicative of a robust gastrointestinal colonization in human volunteers. Immunogenicity studies indicated that ~60% of the vaccinated volunteers had significant peak IgA ASCs to LPS, and a proportion of these volunteers ~60%, also

mounted 4-fold or greater rises in serum and urine IgA titer to LPS. Subsequent challenge of immunized volunteers demonstrated complete protection against the severe symptoms of dysentery [30]. These results provided proof of concept for the VirG(IcsA)-based attenuation strategy. In addition, the studies with SC602 in monkeys and humans also indicate that the behavior of live *Shigella* vaccine candidates in rhesus monkeys can be used as a first measure to predict safety and colonization potential, the latter being a critical factor for a robust immune response that can be protective in humans.

The shedding data for WRSS1, WRSS2 and WRSS3 in rhesus monkeys are similar to those seen with SC602 in monkeys as described above. Most of the animals given the *S. sonnei* vaccine strains shed for 48 h and thereafter shedding was gradually reduced in all three groups of monkeys. Although the vaccinated animals in this study were not challenged for efficacy, significant immune responses to Invaplex and to the Ipa proteins were manifested in all 3 groups allowing one to predict that, like WRSS1, that has been extensively tested in human volunteers, WRSS2 and WRSS3 will also colonize human volunteers robustly and as a result demonstrate significant immunogenic responses that were seen with WRSS1. A single dose of 10^3 – 10^6 CFU in U.S. volunteers resulted in excretion of WRSS1 from 82% of the vaccines with 66% of the volunteers shedding WRSS1 till day 7 [31]. In a subsequent dose-escalating outpatient study in Israel, 71% of the volunteers who received one dose of either 10^3 , 10^4 or 10^5 CFU of WRSS1, excreted the vaccine strain with an average of 6 days [33]. Although efficacy results for WRSS1 are forthcoming, the robust colonization of WRSS1 in U.S. and Israeli volunteers paralleled strong and significant mucosal immune responses in immunized volunteers and were of a magnitude (~75% of the vaccinated volunteers had a significant IgA ASC to LPS) that was comparable to that seen in SC602-immunized volunteers who were subsequently protected after challenge with strain 2457T [30,31,33]. Although WRSS1 was not previously tested in rhesus monkeys, one can draw positive conclusions from this study that its ability to colonize and elicit an immune response in primates can be linked to its excretion and immunogenicity profile seen in human volunteers [31,33].

Since WRSS2 and WRSS3 appear to colonize rhesus monkeys to the same extent as WRSS1 (and SC602), and since the immune responses in primates are comparable among the three *S. sonnei* vaccine strains, it is likely that, the new vaccine candidates will colonize the human gut and generate significant immune responses. The results from this primate study are encouraging and support Phase 1 dose-escalating studies of WRSS2 and WRSS3.

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